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The Interaction of pseudomonas toxins with epithelial cell membranes; a primary stage in the pathogenesis sequence of cellular intoxication

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ABSTRACT

The purpose of this work has been to determine the nature of
alterations of the cell membrane which result from its interaction
with and penetration by Pseudomonas exotoxin (PE) or endotoxin or
Diphtheria toxin (DE); and to determine what relation, if any,
exists between the enzymatic function (ADPRase) in the a-fragment
of the PE peptide and its ability to gain entry into a host cell.
The turtle bladder was chosen as the host cell system because its
major function, the reabsorption of Na, Cl, and HCO_3, depends on
readily-measured electrical parameters, e.g. the transepithelial
potential (PD), conductance (G or 1/R), and short-circuiting
current (Isc).

The main findings to this date are: (i) When added to the
mucosal fluid but not to the serosal fluid, the proenzymatic form of
PE (i.e. that with low ADPRase activity) and not the enzymatically-
activated form decreases the Isc, PD, and G of bladders in Na-rich
and Na-free media. (ii) Pre-mixing of PE with a 2:1 molar excess
of anti-PE antibodies effectively blocks the effect of this exotoxin
on the bladder; as does pre-heating (100°C) of the exotoxin. (iii)
In contrast to the inhibitory action of PE, the diphtherial exotoxin
(DE) increases the Isc and G after its addition to either the mucosal
or serosal fluid in a Na-rich system, but only after addition to the
mucosal fluid in a Na-free system.

Interpretation. The whole PE polypeptide (72,000 Daltons)
consists of an enzyme-containing a-fragment (25,000 Daltons) which
is presumably lethal, once inside the cell; and an enzyme-free b-
fragment (47,000 Daltons) which is believed to be responsible for
the membrane binding, translocation and entry of toxin into the cell
fluid. However, we find that the intensity of the toxin-induced
electrical response of the bladder is inversely related to the in-
herent ADPRase activity in the exogenously added, whole PE molecules.
Therefore the interaction (with the membrane) of the b-fragment in
a whole PE molecule is critically dependent on the state of ADPRase
activity in the a-fragment of that molecule. Whether or not the
isolated b-fragment is capable of interacting with the membrane of
any susceptible host cell remains to be shown; and this is one of
our specific aims for the next phase of this research.

I) THE PROBLEM

a) <u>General Aspects</u>. The mortality rate in patients with severe burns (or with malignancy and other diseases) is significantly increased in the presence of superimposed bacterial infections due to Pseudomonas aeruginosa. This bacterium produces a soluble, heat-labile polypeptidic exotoxin which is highly lethal to experimental animals and destroys cells in tissue culture preparations. Although the experimentally-induced disease differs from the clinical disease, there is as yet no a priori reason to deny a critical pathogenetic role of this exotoxin in human disease states.

b) <u>Specific Aspects</u>. On one hand, the toxin-induced cause of cellular destruction and death is ascribable to an inherent NAD-dependent ADPR transferase activity which ribosylates the elongation factor-2 and blocks protein synthesis. On the other hand, the mechanism whereby a macromolecule of this size (66,000 Daltons) penetrates the plasma membrane to gain entry into the cell is not yet fully understood.

<u>Our problem</u> has been to determine how the exotoxins of Pseudomonas aeruginosa, C. Diphtheriae and others interact with and alter the permeability of the plasma membranes in order to gain entry into the cells of the isolated turtle bladder epithelium. The measured signals of these interactions are changes in the transepithelial potential (PD), short circuiting current (Isc) and conductance (G), which are monitored with better than a second-to-second resolution in time. We now know that the toxin-induced changes in these parameters begin within one minute and last for as long as the experiment (5-6 hours) and that these changes are evoked after administration of the proenzymatic form of Pseudomonas toxin, <u>not</u> after that of the enzymatically-activated state of the same exotoxin. This is in harmony with data of others on the binding and cytotoxicity of Pseudomonas and Diphtherial exotoxins to cells in various tissue culture lines.

The <u>present problems</u>, which will be studied during the 1978-79 period, emerge from

(a) The ability of the PE receptors on the apical membrane of the turtle bladder to discriminate between the proenzymatic and enzymatically-activated forms of the same PE molecule; from

(b) the difficulty in dissociating the membrane component from the intracellular component of the action of the bacterial toxins on the basis of the time-dependent course of the toxin-induced changes in the permeability and transport parameters of the turtle bladder; and from

(c)   the difficulty in dissociating interactions of the enzymatic fraction (a or A) from those of the enzyme-free fraction (b or B) of the intact PE and DE molecules respectively.

The plans for attacking these problems are given in section V of this annual report and in the proposal for renewal of this contract.

The background, the difference between our approach and that of others in this field, the tentative hypotheses and experimental plans will be presented below.

II) APPROACHES

Up to now, the approach used to determine the mechanism of translocation of extracellularly-located bacterial exotoxins (from C. Diphtheria and Pseudomonas aeruginosa) through the plasma membrane and into the cellular interior has been to study the uptake ^{125}I-labelled or non-labelled toxin into the cells of various tissue culture lines. This "uptake" includes the recognition-binding of toxin by membrane receptors, the intra-membrane flow, and the delivery of toxin into the cell.

The work of Collier, Pappenheimer, Iglewski and several others indicate that each of these toxins (DE and PE) is polypeptide (62,000 and 72,000 Daltons) which can be cleaved proteolytically into two fragments; A and B for diphtheria toxin (DE) and a and b for Pseudomonas toxin. The isolated a- or A-fragment, which contains the NAD-dependent ADPR transferase, is toxic if in the cytoplasm but not if located in the extracellular fluid of animals or tissue culture cells. On the other hand, the b or B fragment, which is devoid of enzyme, is required for membrane penetration and cell entry of whole toxin.

An ingenious approach, used by Uchida, Pappenheimer and Harper and by Nelson and Gill, was to study the enzymatic activity (ADPRase) and cytotoxicity of non-toxic, mutant forms of Diphtherial exotoxin, i.e. toxin-like proteins (CRM's) which are immunologically related to DE. Two of these (CRM_{30} and CRM_{45}) contain normal A and mutant B fragments while another (CRM_{197}) contains an enzymatically-inactive A and a normal B fragment. Cytotoxicity was induced by combining A of CRM_{45} with either B of CRM_{197} or with B of the non-mutant toxin.

Our approach to the study of membrane binding and transmembrane flow of toxin differs fundamentally from the aforementioned approaches with respect to the nature of the observed parameters. We are measuring the effects of the toxin-to-membrane interaction on the electrical permeability and ion flows across the toxin-altered membrane. The other approach (used mainly on suspensions of cells from tissue culture) entails measuring the cytotoxicity or the toxin-to-membrane interaction per se-- without determining exactly what this interaction does to the physiological activity of the toxin-occupied membrane.

The rationale underlying our approach using turtle bladder epithelium (which is essentially that originally used in frog skin by Ussing in 1948) is that the ion-selective permeability or the electrical conductance (G), the rates of active ion transport (e.g. reabsorption of Na, HCO_3, and Cl), the short-circuiting current (Isc) and the potential difference (PD) reflect the states of the plasma membranes under various physiological conditions and the induced changes in these states under conditions which prevail during inhibition of ion transport (e.g. ouabain, amiloride, or acetazolamine) or under conditions which prevail during acceleration of ion transport (e.g. by norepinephrine or cyclic AMP).

The application of this approach to the study of membrane interaction with bacterial toxins has not only shown the sensitivity of electrophysiological methods, but has provided a useful dimension for gaining insight into the means by which macromolecules (like toxins) can bind to and traverse a plasma membrane barrier.

III) RESULTS

The results obtained up to now deal mainly with the effects of the Pseudomonas toxin on the permeability and ion transport parameters of the isolated, short-circuited bladder bathed in Na-rich and Na-free media. What follows deals with: the unique sidedness, irreversibility and heat-lability of the toxin effects; and more specifically with the activation state of the inherent toxin enzyme.

Mucosal-sidedness. In the first set of experiments, it was found that exposure of the mucosal surface to nanomolar concentrations of Pseudomonas exotoxin was followed by rapidly developing and significant changes in the transport parameters (Isc, PD, and R) of five half-bladders in Na-rich and of five mated half-bladders in Na-free media. On the other hand, exposure of the serosal surface to the same or larger concentrations of this toxin failed to induce detectable changes in Isc or Pd.

Figure 1 shows the effect of this Pseudomonas exotoxin on the Isc and PD of one of the half-bladders in a Na-rich bathing system. Within 3-4 minutes after the mucosal addition of toxin, the Isc (and PD) began to increase, reached a maximal level in 12 minutes, then declined rapidly $[t(\frac{1}{2})\approx 7\ min]$ to a new steady state level which was 40% less than that of the control period. The PD and conductance were also decreased. These results were replicated in 15 experiments on bladders in Na-rich media, the mean values for which are shown in Table 1.

On the basis of these data, it can be inferred that the exotoxin interacts with the apical membrane to block the passive entry of Na into the cell. Under the same Na-rich bathing conditions, it has previously been shown that the Isc is approximately equal to the net rate of active Na reabsorption (Gonzalez). However, the presently observed effects of exotoxin were not restricted to the Na pathways of the apical membrane; changes involving the anion pathways were also found (Figure 2) in half-bladders bathed in Na-free media.

Figure 2 shows the effect of exotoxin on Isc and PD of one of the half bladders in Na-free bathing media. After the mucosal addition of exotoxin, the Isc and PD decreased rapidly $[t(1/2)\approx min]$ to near-zero levels and remained at near-zero for the duration of the experiment. The transepithelial electrical conductance was also decreased. Under the Na-free conditions, the orientation of the PD and Isc (serosal side negative) was opposite to that of bladders in Na-rich media. The magnitude of such a negative Isc has been shown to approximate the sum of the net reabsorption of HCO_3 and Cl; and these anion pump elements probably reside in the apical membrane. Therefore the pseudomonas exotoxin interacts with the apical membrane to block the entry of the anions (HCO_3 and Cl) as well as that of sodium into the cell fluid.

Crude-dose response. A semi quantitative dosage-response pattern was obtained from data of 4 experiments on paired half-bladders in Na-rich media. Detectable decreases in Isc (to 85% of the control level in 10 min) were found in two experiments when the mucosal concentration of toxin was as low as 5×10^{-10}M. Maximal decreases (to 60% of the control level) were found when this concentration was increased to 5×10^{-9}M.

Irreversibility. Shown in Figure 1 and in four other experiments (not shown) are data on the effect of repeated removals of the toxin-containing fluid and replacements with toxin-free fluid. Once the Isc and PD reach the lower steady levels in the toxin-treated bladders, the removal of toxin fails to result in a restoration of these parameters to control levels; and the subsequent addition of a potent toxin produces no further decrease in Isc and PD.

Heat-lability. The heat-labile nature of the Pseudomonas exotoxin was tested with respect to its effect on the sodium transport parameter (Isc of a bladder bathed in Na-rich media). The mucosal addition of a pre-heated (100°C) aliquot of toxin produced no detectable change in the Isc or PD. But the subsequent addition of a non-heated (25°C) aliquot of the same exotoxin was followed by the expected toxin-induced changes in Isc and PD (Figure 3). This shows that the toxin-induced changes are not those of any non-specific protein. However one cannot determine whether such a heat-inactivated toxin fails to bind to or penetrate the apical membrane, which means that the heat-lability of this toxin is non-specific in this respect. Pre-heating of this toxin has also been shown to eliminate its lethal effects in whole animals, its cytotoxic effects in cultured cells, and its intrinsic ADPR transferase activity. We then decided to test for a more specific property of Pseudomonas toxin, namely its reactivity with antiPseudomonas toxin antibodies.

Immunoreactivity. The objective of these experiments was to determine whether a specific antibody against the exotoxin of this strain (PA-103) of Pseudomonas aeruginosa would block its effect on ion transport. Therefore the exotoxin was pre-mixed with a twofold molar excess of specific anti-PA-103 antibodies in serum from immunized rabbits. The addition of this antibody:toxin mixture to the mucosal fluid of one half-bladder effectively blocked most of the changes in Isc and PD that would have occurred after the addition of toxin alone, as was found after the subsequent addition of toxin alone to the same half-bladder (Figure 4); and as was found after addition of the same toxin to the mucosal fluid of the mated half-bladder (not shown). The protective effect of this twofold excess of antibody against the inhibitory effect of toxin alone was found in two of three similar experiments on paired bladder halves. In two other experiments, it was found that pre-mixing of equivalent quantities of non-immune serum or bovine serum albumin with toxin did not protect against the toxin-induced decreases in Isc and PD.

Erratic results. The next set of experiments, performed with other lots of purified and lyophilized exotoxin, yielded erratic results. The exotoxin in these lots had been stored in the form of dried powder (300 µgm aliquots) in each of 12 vacuum-sealed vials. The addition of 20 to 100 µgm doses of toxin from 6 of these vials to the mucosal fluid of 6 half-bladders in Na-rich media was followed by the usual transient increase and subsequent decrease in Isc and PD (See Figures 1,3). However the addition of equimolar or greater quantities of toxin from 6 other vials to 6 other half bladders under identical conditions produced no detectable changes in the Isc or PD--even though the toxin in each of the first set of vials was made from the same bacterial source as that in each of the second set of vials.

State of activation of ADPR transferase. It appeared possible that the electrical responses and the lack of such responses in these bladders could be related to variations in the ADPR transferase activity in the various toxin samples that had been used. This is because the toxin-induced ribosylation of elongation factor 2 in lysates of rabbit reticulocytes is increased several fold by pre exposure of that toxin to dithiothreitol (DDT) plus urea. Such a transformation implies the existence of two states of the toxin-enzyme: (i) that which is potentiated by DTT and urea, a "proenzymatic" state; and (ii) that which is in a potentiated state even before exposure to DTT and urea, an "enzymatically-activated" state of the same toxin.

Therefore we determined the ADPRase activity of lyophilized toxin preparations using $[^{14}C]$·NAD as the substrate and a wheat germ preparation as the source of elongation factor. The enzymatic activity of each aliquot of toxin was determined before and after the addition of dithiothreitol (DTT) and urea or before and after the toxin preparation was frozen to -70°C and thawed to 25°C.

The activity of toxin which had produced little or no effect on the Na transport parameter was just as high in the absence as it was in the presence of DTT and urea; and just as high before freezing and thawing as it was after this maneuver. On the other hand, the activity of toxin which had produced a distinct decrease in Na transport was increased fivefold after the addition of DTT and urea or after the freezing and thawing procedure.

It is not yet known what steps in the purification or lyophylization can cause a spontaneous increase (to maximal levels) in the ADPR-transferase activity of these pseudomonas exotoxins. Nevertheless, on the basis of these data (not shown), it was inferred that: (i) the proenzymatic state of Pseudomonas exotoxin is a requirement for its interaction with the apical membrane and consequent entry into the bladder epithelial cells; and that (ii) if this proenzymatic state be converted to the enzymatically-activated state, the same exotoxin will no longer interact with the putative receptor sites on the apical membrane of the bladder cell. These

predictions were then verified in the next set of experiments, using paired samples of a proenzymatic toxin preparation. Two aliquots of this preparation were exposed to DTT + urea and two others were not so exposed. The ADPR transferase of the (DTT + urea)-treated toxin was shown to be increased sixfold over that of untreated toxin. Next, the addition of an aliquot of (DTT + urea)-treated toxin to the mucosal fluid produced small, spontaneously reversible changes in the Isc (and PD) of an isolated turtle bladder in Na-rich media. The subsequent mucosal addition of the untreated aliquot of the same toxin (which had not been exposed to urea and DTT) to the same bladder was followed by clear-cut, sustained decreases in the Isc, PD and conductance (Figure 5). The addition of DTT + urea alone was shown to be without effect on the electrophysiological parameters. It was then found that the enzymatic state of the same proenzymatic toxin was activated after three consecutive freezes to -70°C and thaws to 25°C (i.e. without addition of extraneous compounds such as DTT and urea). Not shown is the fact that the addition of an aliquot of the consecutively frozen and thawed toxin to the mucosal fluid of a bladder in Na-rich media was without effect on the Isc or PD, and then the subsequent mucosal addition of the untreated aliquot of the same toxin to the same bladder was followed by the expected decreases in Isc, PD and conductance.

In order to gain further insight into the specificity of the interaction of Pseudomonas toxin with the plasma membrane(s) of the turtle bladder epithelial cell, bacterial toxins other than the exotoxin of Pseudomonas (e.g. Diphtherial exotoxin, Pseudomonas endotoxin, and Cholera toxin) were added to the bathing fluids of short-circuited bladders in Na-rich or Na-free media.

Diphtherial Exotoxin. Within five minutes after the addition of Diphtherial exotoxin to the mucosal fluid (10^{-8} M) of a half-bladder in Na-rich media, the Isc and conductance (1/R) increased rapidly [$t(\frac{1}{2}) \approx$ 5 to 7 min.] to reach levels that were 60% higher than control levels in 20 minutes. A secondary, more gradual rise [$t(\frac{1}{2})$ 30 min.] in Isc and conductance began 1½ hours after toxin addition; this secondary rise continued for another 1½ hours until the levels of Isc and conductance were 75% greater than control levels. The concomitant addition of DE to the serosal fluid of the mated half bladder was followed by a latent period of 1 hour after which the Isc and PD increased at a moderately rapid rate [$t(\frac{1}{2})$, 30 min.] and reached the same level (175% greater than control) as was reached in the mated half-bladder. The PD remained constant after either the mucosal or the serosal addition of DE. These data can be explained by assuming that Diphtherial toxin reaches receptor sites along the Na-selective paths on the cytoplasmic-facing surface of the apical membrane.

However the Diphtherial toxin was found to induce changes in the anion transport as well as in the Na transport parameter of the bladder. Thus after addition of DE to the mucosal fluid

$(10^{-8}M)$ of bladders bathed by Na-free media, the levels of Isc and PD increased (after a latent period of less than 1 minute) to twice those in the control period while the conductance remained unchanged. These increased levels returned rapidly to control levels after replacement of the mucosal fluid with toxin-free mucosal fluid. No change in any of these anion transport parameters was found after the serosal addition of DE. This suggests that DE interacts directly with receptor sites along the anion-selective, pump-containing paths on the mucosal fluid-facing surface of the apical membrane.

A general yet tentative accounting for the effects of DE on both the sodium and on anion transport parameters would require the following assumptions: (i) This toxin is capable of entering the cytoplasmic compartment by penetrating the apical or the basal-lateral membrane from the adjacent external fluid. (ii) There are at least two discrete sets of DE receptor sites; one on the cytoplasmic-facing surface of the apical membrane in the vicinity of the Na-selective paths and the other on the mucosal-facing surface of this membrane in the vicinity of the anion pump elements.

Pseudomonas Endotoxin. The electrophysiological response to the mucosal addition of Pseudomonas endotoxin (a lipopolysaccharide) differed from that to either of the polypeptidic exotoxins. Within two minutes after addition of the endotoxin to the mucosal fluid ($\sim 5\mu gm/ml$) of bladders in either Na-rich or Na-free media, the Isc and conductance increased rapidly $[t(\frac{1}{2})=10 \text{ min.}]$; and in 30 min. reached levels that were 35% higher than control levels. No such effects were found after addition of the endotoxin to the serosal fluid of bladders in Na-rich or in Na-free media. Evidently the receptor sites for this endotoxin are located in the apical membrane; but the data are not yet sufficient to ascertain whether these sites are on the cytoplasmic-facing or the mucosal-facing surface of this membrane.

IV) CONCLUSIONS

The means by which lethal bacterial exotoxins (Diphtheria, Pseudomonas, and other organisms) cross the plasma membranes and gain access to the cytoplasm of a host cell has been a major unsolved problem in the pathogenesis of the cellular destruction in toxin-invaded cells. Currently available data of others are sufficient to show: the kinetics of toxin binding to receptor sites on the plasma membrane and the kinetics of uptake of toxin by cells which have been exposed to such toxins. However, none of these data is sufficient to indicate what happens to the fundamental physiological properties of a toxin-invaded membrane, i.e. properties such as active ion transport and ion-selective permeability which are critical for optimal cellular function and survival.

Our data on the epithelial cell layer of turtle bladders provide new insight into the physiological consequences of the interactions of Pseudomonas and Diphtherial exotoxins with the apical membrane of these cells. We can now draw tentative inferences about what happens to these membrane after they have been exposed to the aforementioned toxins.

1. The proenzymatic state and not the enzymatically-activated state of Pseudomonas exotoxin (PE) interacts with receptor sites on the surface and/or within the apical membranes of the turtle bladder epithelium. The consequences of this interaction are:

    (a) lowering of the Na-selective conductance of the apical membrane, thereby retarding the transapical and transcellular flow of sodium;

    (b) lowering the potential energy (driving force) of the anion pump elements in the apical membrane, thereby retarding the transapical and transcellular flow of anions (Cl and HCO_3), without appreciably changing the anion-selective conductance of this membrane.

2. Since specific anti-PE-antibodies protect against the toxin-induced inhibition of Na transport as well as against the toxin-induced ADP-ribosylation of elongation factor 2, it appears that fragment b (the 45,000 Dalton penetrant moiety of the toxin peptide), as well as fragment a (the 21,000 Dalton, ADPRase-containing fragment), interact with the specific PE-antibodies.

3. The Diphtherial exotoxin (DE) also interacts with receptor sites on the apical membrane of these cells. But in contrast to PE, which only penetrates the apical membrane, DE can penetrate either the apical or the basal-lateral membrane to enter the cell (see item 4, below). The consequences of DE : membrane interactions (distinct from those of PE) are:

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(a) to <u>increase</u> the Na-selective conductance of the apical membrane, thereby <u>accelerating</u> the transapical and transcellular flow of Na. The potential of the Na pump, located in the basal-lateral membrane, is increased due to the accumulation of cellular Na secondary to the accelerated flow of this ion across the toxin-altered apical membrane.

(b) to <u>increase</u> the anion-selective conductance of the apical membrane, thereby accelerating the <u>transapical</u> and transcellular flow of anions--without <u>appreciably</u> changing the anion pump potentials in this membrane.

4.   The DE-induced acceleration of Na transport is evoked after exposure of either the mucosal surface (rapid onset) or the serosal surface (slow onset) to this toxin. In contrast, the DE-induced acceleration of anion transport is evoked only after exposure of the mucosal surface (rapid onset) of this toxin and this transport rate promptly returns to control levels when the toxin is washed out or diluted.

Therefore, it is inferred that DE receptors in the neighborhood of Na-selective paths are located on the cytoplasmic-facing surface of the apical membrane while DE-receptors in the neighborhood of the anion-selective paths are located on the mucosal fluid-facing surface of the apical membrane.

V) PROBLEMS AND PLANS

(1) In view of the current hypothesis which holds that the b-fragment of PE (or the B-fragment of DE) is required for membrane binding and translocation of the whole polypeptide complex (AB), we plan to determine the effects on ion transport parameters of some of the available non-toxic mutant forms of DE. For example, CRM_{30} or CRM_{45}, which contains normal A and defective B, should be without effect on ion transport. On the other hand CRM_{197}, which contains normal B and defective A might well produce changes in ion transport like those produced by the "native" toxin.

(2) Since the proenzymatic form of PE is the "transport-effector" form of this toxin, we plan to see whether or not the pro-enzymatic form of DE is likewise an ion transport effector form. This will be of particular interest in the case of DE because of the availability of the non-toxic mutant forms of this toxin (See item 1 above).

(3) In order to separate the membrane effects from the intra-cytoplasmic effects of the bacterial toxins, we plan to determine their effects on the transmembrane fluxes of Na, Cl, or H in isolated vesicles drived from the apical membranes of the turtle bladder epithelial cells. To do this experiment, we have to separate the apical from the basal-lateral membranes by free-flow electrophoresis (Hannig method). This technique has been applied to turtle bladder and renal epithelia by Dr. Rolf Kinne who has col-laborated with us recently.

If we do obtain toxin-induced effects on transport in these vesicles, this would constitute a direct determination of membrane changes in the absence of any cytoplasm.

(4) We plan to determine the immunospecificity of DE-induced and PE-induced effects on transport. Although we have found the PE-induced inhibition of Na transport is effectively blocked by anti-PE-antibodies, we have yet to determine that this inhibition is not blocked by other antibodies, i.e. by anti DE antibodies. Thus we plan to do cross-reaction tests in which DE will be pre-mixed with anti PE antibodies or with anti DE antibodies and in which PE will be pre-mixed with anti DE- or anti PE-antibodies prior to addition of these antibody-antigen mix-tures to the bathing fluids of the turtle bladder preparations.

These tests will be supplemented by additional ones on protection afforded by various molar ratios of antibody to antigen in each case.

(5) Other plans which have been outlined in the first research proposal deal with the following items:

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(a) Dosage-response data

(b) Effects of toxins on (Na + K) ATPase, adenylate cyclase, protein kinase or other membrane-bound transport-related enzymes or proteins

(c) Parameters of binding of toxins to various sub-cellular fractions of turtle bladder cells (e.g. cytosolic proteins, membrane fragments, mitochondria, nuclei)

(d) Nature of the chemical bonds between toxin and membranes (in the whole intact tissue and in the isolated state) as can be determined from the effect (on toxin-induced changes) of pH and various group-specific reactive agents (e.g. disulfonic stilbenes, hydroxylamine and others

(6) Effects of other exotoxins will be determined--e.g. pilot studies have been started with the exotoxin of V. Cholerae and the endotoxin of Pseudomonas.

SELECTED LITERATURE CITATIONS

We cite here some several significant advances in (a) Methodology (b) bacterial toxin effects and (c) ion transport phenomena.

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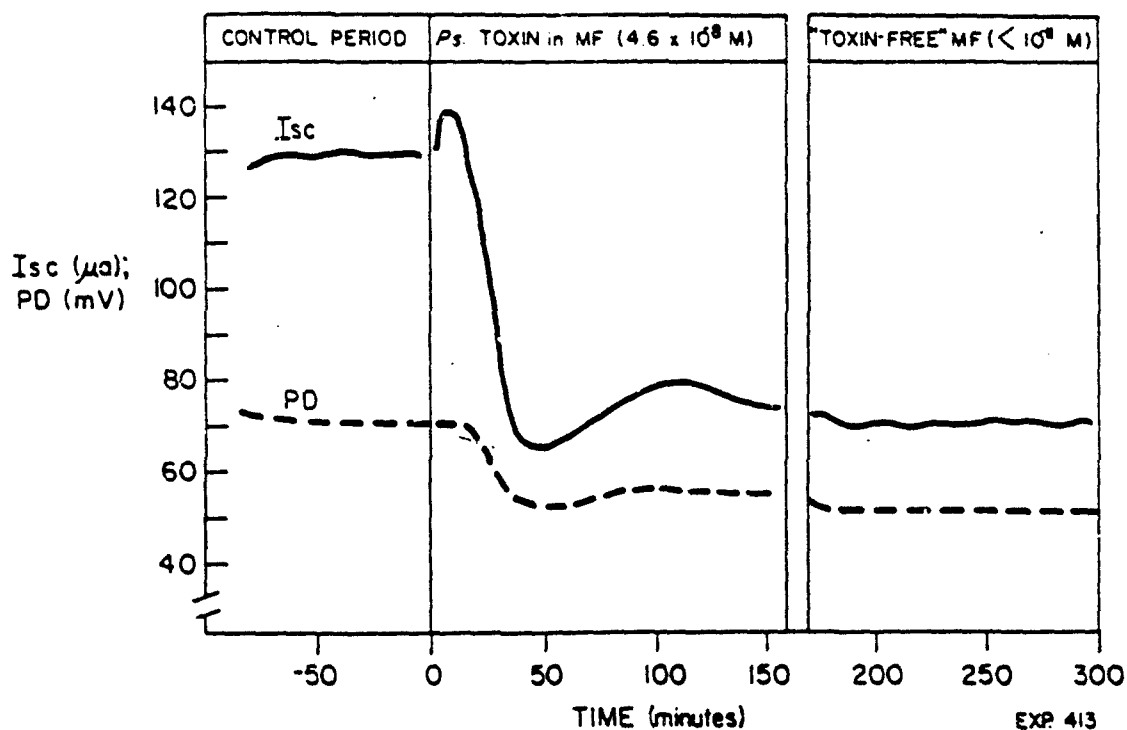J. Physiol. (London), In Press, 1978

Figure 1. Effect and irreversibility of effect of PA-103 exotoxin on I_{sc} and PD of a turtle bladder in a Na-rich bathing system Mucosal fluid. Na_2SO_4 Ringer (Cl-free, HCO_3-free). Serosal fluid Na Ringer (with Cl and HCO_3).

Table 1. Mean values ($\pm$SE) for Isc, PD, and 1/G before and after mucosal addition of PA-103, 10^{-8}M, to 15 bladders in the Na-rich bathing system; and means ($\pm$SE) of the individual toxin-induced changes in these parameters (MPD).

	Isc (Na)	PD (mv)	1/G=R (Kohms)
Before	93.0$\pm$11.5	72.1$\pm$5.3	0.91$\pm$0.11
After	50.4$\pm$6.2	51.8$\pm$1.7	1.20$\pm$0.20
MPD	-44.6$\pm$2.9	-29.5$\pm$2.5	+22.7$\pm$2.6

All values of MPD were significantly different from zero (i.e. $P<0.001$).
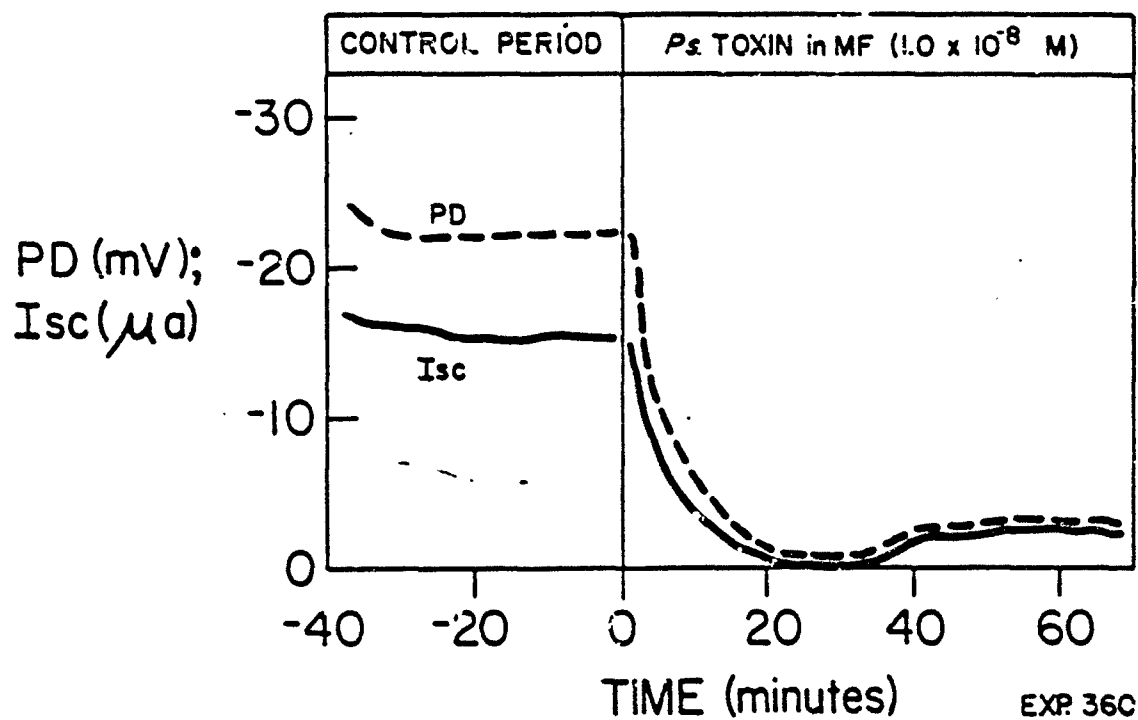
Figure 2. Effect of PA-103 exotoxin on I_{SC} and PD of a turtle bathed on both surfaces by identical Na-free (choline) Ringer containing Cl and HCO_3.
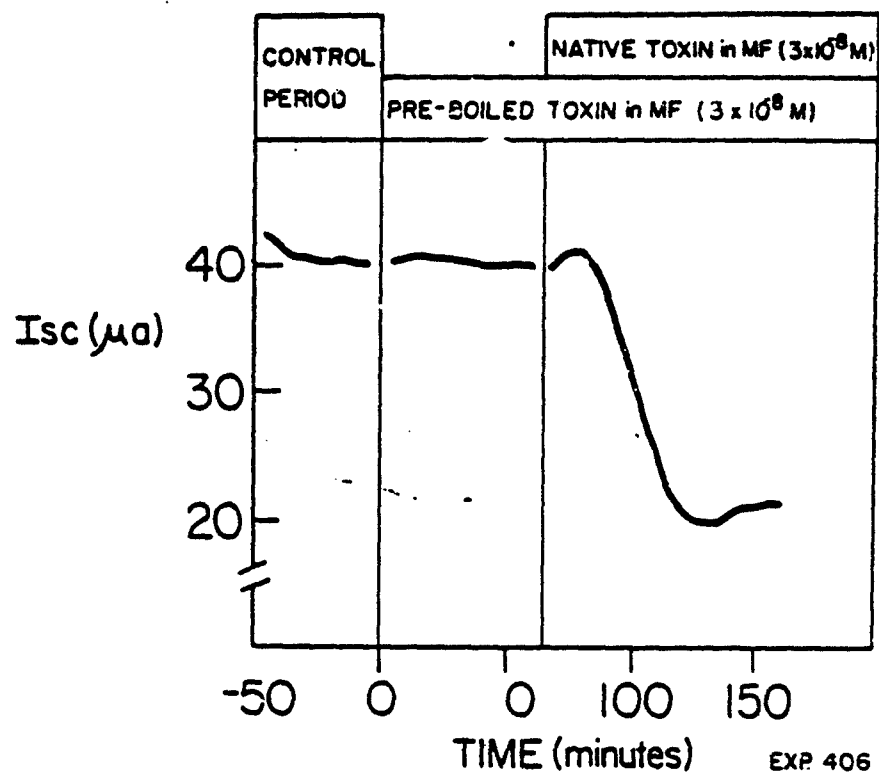
Figure 3. Lack of effect of pre-boiled PA-103 exotoxin on the I_{sc} of a turtle bladder in the Na-rich bathing system (described in the legend of figure 1).
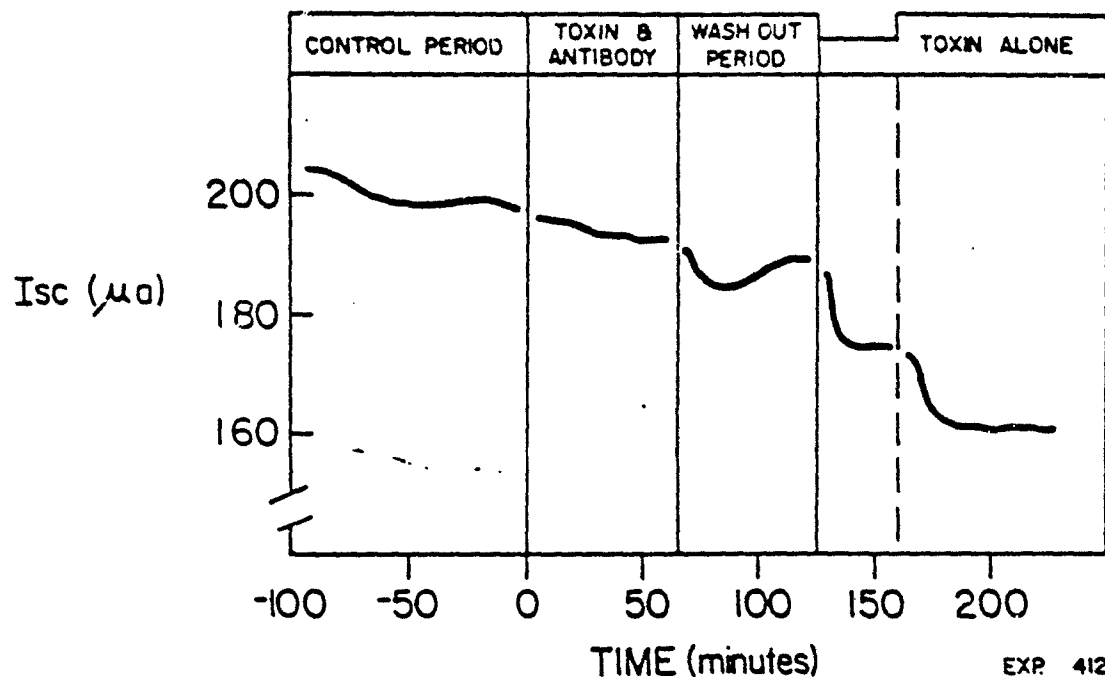
Figure 4. Protective effect of specific antibodies against PA-103 exotoxin and the subsequent effect of PA-103 exotoxin alone on the I_{sc} of a bladder in the Na-rich bathing system.
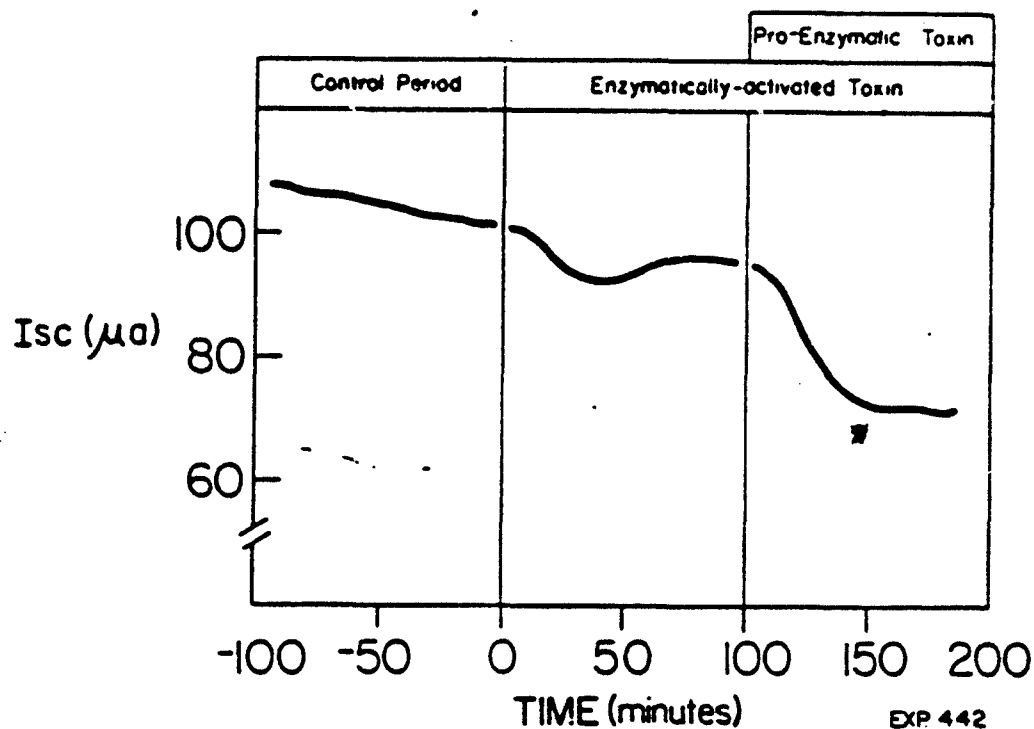
Figure 5. Lack of effect of enzymatically pre-activated PA-103 (DTT, urea) and subsequent effect of the proenzymatic form of the same toxin on the I_{sc} of a bladder in the Na-rich bathing system.